

December 2001

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EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 33, No. 4, 226-233, December 2001

# Molecular mechanisms involved in human platelet aggregation by synergistic interaction of platelet-activating factor and 5-hydroxytryptamine

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Accepted 24 November 2001

Abbreviations: ($Ca^{2+}$); calcium, COX, cyclooxygenase; ERK1/2, extracellularly regulated mitogen-activated protein kinases; 5-HT, 5-hydroxytryptamine; NO, nitric oxide; PAF, platelet-activating factor; PKC, protein kinase C; PLC, phospholipase C; PI 3-kinase, phosphatidylinositol 3-kinase; $TXA_2$, thromboxane $A_2$

## Abstract

**Our recent studies have shown that co-activation of $G_q$ and $G_i$ proteins by 5-hydroxytryptamine (5-HT) and adrenaline show synergism in human platelet aggregation. This study was conducted to examine the mechanism(s) of synergistic interaction of 5-HT and platelet activating factor (PAF) in human platelets. We show that PAF, but not 5-HT, increased platelet aggregation in a concentration-dependent manner. However, low concentrations of 5-HT (2 μM) potentiated platelet aggregation induced by sub-threshold concentration of PAF (40 nM) indicating a synergistic interaction between the two agonists and this synergism was blocked by receptor antagonists to either 5-HT or PAF. 5-HT also potentiated the effect of PAF on thromboxane $A_2$ ($TXA_2$) formation and phosphorylation of extracellularly regulated mitogen-activated protein kinases (ERK1/2). The synergism of 5-HT and PAF in platelet aggregation was inhibited by calcium ($Ca^{2+}$) channel blockers, verapamil and diltiazem, phospholipase C (PLC) inhibitor, U73122, cyclooxygenase (COX) inhibitor, indomethacin, and MEK inhibitor, PD98059. These data suggest that synergistic effect of 5-HT and PAF on human platelet aggregation involves activation of PLC/$Ca^{2+}$, COX and MAP kinase pathways.**

**Keywords:** platelet aggregation, PAF, 5-HT, MAP kinase, synergism

## Introduction

Platelets play an important role in maintaining the vascular integrity and haemostasis. Upon vascular damage, platelets undergo rapid changes; become more spherical, extrude pseudopodia and activate their fibrinogen receptors leading to aggregation. During this process, platelets release granule contents and substances that act in autocrine fashion to further enhance aggregation (Siess, 1989; Brass *et al*., 1993). We, and others have shown that various platelet agonists at low concentrations elicit synergistic interactions (Ware *et al*., 1987; Shah and Saeed, 1995; Saeed *et al*., 1997; Masini *et al*., 1998; Shah *et al*., 1999; Francesconi *et al*., 2000). However, the molecular basis of such an interaction is not well understood.

Most of the platelet agonists, like thrombin, ADP, PAF, epinephrine and 5-HT, interact with their transmembrane receptors on platelets coupled to GTP binding proteins (G proteins). The G-proteins mediate a variety of cellular processes by activating different effector molecules, including adenylyl cyclase, inositol phospholipid-specific phospholipase C (PLC) or ion channels (Siess *et al*., 1989; Exton, 1996). In platelets, stimulation of receptors coupled to $G_q$ protein (e.g., by 5-HT, PAF or thrombin) leads to activation of PLC and thus generation of second messengers, diacylglycerol (DAG) and inositol-1,4,5-triphosphate ($IP_3$), which results in the activation of protein kinase C (PKC) and the mobilization of intracellular $Ca^{2+}$, respectively (Obberghen-Schilling and Pouyssegur, 1993). Both $Ca^{2+}$ and PKC stimulate platelet aggregation and also elicit synergism in platelets (Crabos *et al*., 1992). Consistent with the potential involvement of $G_q$/PLC pathway, the deficiency of $G_q$ protein in transgenic mice leads to impairment of agonist-induced platelet aggregation (Offermans *et al*., 1997).

PAF, a phospholipid mediator, is a very strong platelet activator and human platelets show high affinity binding sites for this agonist. It also induces adhesion of platelets to the endothelium in the presence of activated leukocytes (Hirafuji and Shinoda, 1991). PAF is also known to play an important role in various patho-

physiological conditions that include modulation of blood pressure, hypotension, cardiac dysfunction in cardiac anaphylaxis, and hemorrhagic, traumatic, and septic shock syndromes (Anderson *et al*., 1991; Montrucchio *et al*., 2000). Because of its ability to stimulate endothelial migration and angiogenesis, a potential role of PAF in atherosclerosis was suggested (Montrucchio *et al*., 2000). PAF is also known as a potent stimulator of thromboxane $A_2$ ($TXA_2$) production in human platelets.

Another platelet agonist, 5-hydroxytryptamine (5-HT), is released by aggregating platelets at the site of vascular damage and this process can be augmented by PAF (Bailey *et al*., 2000). 5-HT is widely distributed in the body and sub-serves many functions. The type 2 receptors for 5-HT (5-$HT_2$) mediate many physiological functions that include increase in arterial constriction, modulation of perception, mood, feeding behaviour, and platelet aggregation (Roth *et al*., 1998; Robertson, 1991). Very little 5-HT is free in plasma, most being stored in dense granules of platelets. However, local platelet activation and subsequent 5-HT release can present free 5-HT to peripheral tissues that can contribute to a range of cardiovascular problems, including portal hypertension (Robertson, 1991) and vasoconstriction (Roth *et al*., 1998). High plasma 5-HT levels are found in primary pulmonary hypertension (Kereveur *et al*., 2000) and in patients with bladder cancer (Pawlak *et al*., 2000). Similarly 5-$HT_{2A}$ receptor densities tend to increase in depression (Mendelson, 2000). Like PAF, 5-HT also shows mitogenic effects in cardiovascular system (Koba *et al*., 2000). It enhances the atherogenic and mitogenic effects of low-density lipoproteins (LDL) in aortic smooth muscles (Koba *et al*., 2000). Combined $TXA_2$ and 5-$HT_2$ receptor blockade is proposed to prevent coronary artery thrombosis (Willerson et al., 1990).

PAF enhances vasoconstriction of the coronary arterioles (DeFily *et al*., 1996) and at the inflammatory coronary lesions *in vivo* by itself as well as in a synergistic manner with 5-HT (Kozai *et al*., 1997). Because of the close interaction between the two agonists (PAF and 5-HT) and their importance in thrombosis, hypertension and atherosclerosis, this study was conducted to examine the mechanism(s) of synergism between 5-HT and PAF during platelet aggregation. We show that synergistic interaction of 5-HT and PAF is mediated through PLC/$Ca^{2+}$ and cyclooxygenase pathways and is modulated by nitric oxide.

## Materials and Methods

### Materials

PAF, 5-HT, cyproheptadine methysergide, indomethacin, diltiazem, verapamil, chelerythrine and wortmannin were purchased from the Sigma Chemical Co. (St. Louis, MO. USA). U73122 and SNAP were from Alexis LC Labs (UK). All other chemicals were of the highest purity grade available.

### Preparation of human platelets

Blood was taken by venous-puncture from normal human volunteers reported to be free of medication for one week. Blood samples were mixed with 3.8% (w/v) sodium citrate solution (9:1) and centrifuged at 260 *g* for 15 min at 20°C to obtain platelet rich plasma (PRP). Platelet count was determined by phase contrast microscopy and all aggregation studies were carried out at 37°C with PRP having platelet counts between 2.5 and $3.0x10^8$/ml of plasma (Saeed *et al*., 1997).

### Measurement of platelet aggregation

Aggregation was monitored using a Dual-channel Lumi-aggregometer (Model 400 Chronolog Corporation, Chicago, USA) using 0.45 ml aliquots of PRP. The final volume was made up to 0.5 ml with the test drug dissolved either in normal saline or appropriate vehicle known to be devoid of any effect on aggregation. Aggregation was induced with PAF and sub-threshold concentration determined. To obtain the synergistic effect of PAF and 5-HT, we added low concentrations of these agonists. The anti-aggregatory effects of different compounds were studied by pretreatment of PRP with various inhibitors for one min followed by addition of the sub-threshold concentrations of PAF and 5-HT. The resulting aggregation was recorded for 5 min after challenge by the change in light transmission as a function of time. Once the anti-platelet activity of various inhibitors against agonists was established, dose-response curves were constructed to calculate the $IC_{50}$ values of the agonists and inhibitors.

### Thromboxane formation in platelets

Arachidonic acid metabolism and thromboxane A2 ($TXA_2$) formation were studied as described previously (Saeed *et al*., 1997). For these studies, human blood platelets were routinely obtained in plastic bags containing 30-40 ml of concentrated PRP from The Aga Khan University Hospital Clinical laboratory, Karachi.

### Immunoblot analysis of ERK1/2

Platelets were stimulated with PAF at 37°C, lysed with an equal volume of 2 X Laemmli's sample buffer containing 5% β-mercaptoethanol. The samples were heated at 95°C for 5 minutes, electrophoresed on SDS-PAGE (10%) gels and transferred to PDVF nylon membranes. Membranes were incubated overnight with phospho-MAP kinase (p42/44) primary antibody (New England Biolabs) diluted in TBST. Primary antibody was removed and blots washed three times with TBST before adding the horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After

washing six times with TBST, blots were exposed to enhanced chemiluminescence reagent (Amersham Pharmacia) and films developed.

## Results

Treatment of PRP with PAF showed concentration-dependent aggregatory effects on human platelets (Figure 1A). In contrast, 5-HT had no effect on platelet aggregation up to 200 μM (data not shown). But very low concentrations of 5-HT (2 μM) caused marked potentiation of aggregation response mediated by sub-threshold concentration of PAF (40 nM) suggesting a synergism between the two agonists (Figure 1B). To examine the molecular basis of this synergism, we used the selective inhibitors of various signalling pathways. Pretreatment of PRP with 5-HT receptor antagonist,

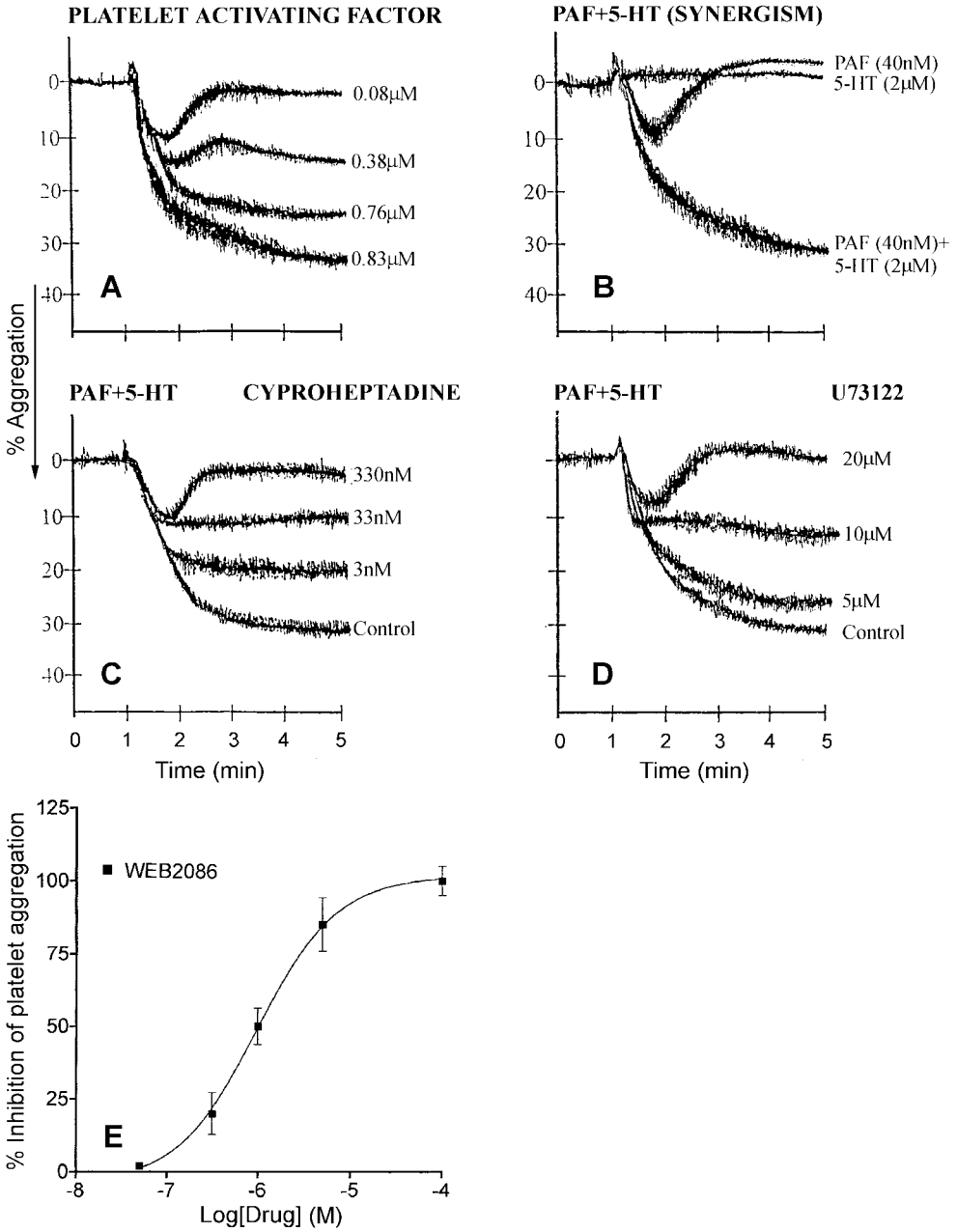


**Figure 1.** (A) Concentration-dependent effects of PAF on human platelet aggregation. PRP was treated with the agonist and platelet aggregation recorded for 5 min. (B) Tracings from representative experiments showing synergism of 5-HT (2 μM) and PAF (40 nM). (C) The synergistic effect of 5-HT and PAF on platelet aggregation is blocked by 5-HT receptor antagonist, cyproheptadine (D) phospholipase C inhibitor, U73122 (E) and PAF antagonist WEB2086. Inhibitors were added one min before the agonists. Control means platelet aggregation induced by 5-HT (2 μM) plus PAF (40 nM) (n=5).

**Table 1.** Effects of various inhibitors on platelet aggregation mediated by synergistic interaction of sub-threshold concentrations of PAF (40 nM) plus 5-HT (2 μM) and PAF alone (800 nM)

| Inhibitors | PAF+5-HT $IC_{50}$ values (μM) | PAF $IC_{50}$ values (μM) |
|---|---|---|
| Cyproheptadine | *4.00 ± 0.02 | *40 ± 4.5 |
| Methysergide | *55.00 ± 2.2 | 52 ± 3.4 |
| WEB 2086 | 0.50 ± 0.08 | 48 ± 5.5 |
| Verapamil | 8.00 ± 0.1 | 20 ± 3.4 |
| Diltiazem | 5.20 ± 0.4 | 15 ± 2.0 |
| PD98059 | 3.00 ± 0.3 | 4.0 ± 0.01 |
| Indomethacin | 0.25 ± 0.001 | 9 ± 1.2 |
| U73122 | 10.00 ± 2.8 | NE |
| SNAP | 0.28 ± 0.04 | ND |
| Wortmannin | 0.62 ± 0.10 | ND |

Data is mean ± SEM ($n$ = 5-7) and is indicated as half-maximal effect ($IC_{50}$) of the inhibitors. (*) Means concentrations in nM. (NE = no effect and ND = not done)

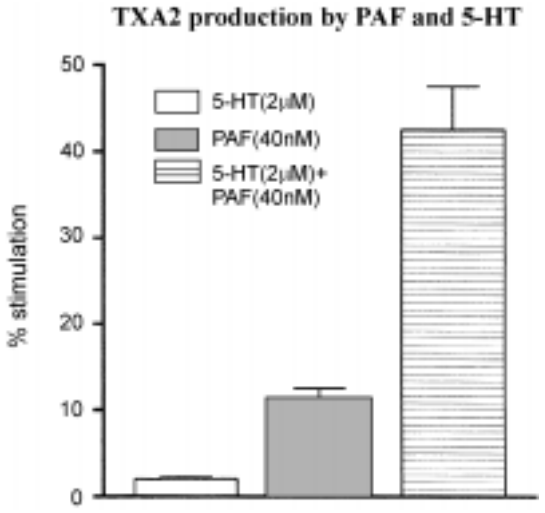


**Figure 2.** Effects of 5-HT and PAF on thromboxane A2 ($TXA_2$) formation in human platelets. Low concentrations of 5-HT (2 μM) potentiate the effect of PAF (40 nM) on $TXA_2$ formation in human platelets (n=7).

cyproheptadine ($IC_{50}$=4nM) was effective in blocking synergism of 5-HT and PAF (Figure 1C). Consistent with the notion that both PAF and 5-HT activate $G_q$/PLC, pretreatment of PRP with PLC inhibitor, U73122, completely inhibited the synergistic effect of PAF and 5-HT with an $IC_{50}$ of 10 ± 3 μM (Figure 1D).

Like cyproheptadine, PAF receptor antagonist WEB 2086 ($IC_{50}$=0.5 μM) also showed marked inhibition indicating that the synergistic effect of 5-HT and PAF was dependent on functional receptors for both agonists (Figure 1E). Since activation of PLC leads to an increase in cytosolic $Ca^{2+}$ due to its release from internal stores by inositol triphosphate ($IP_3$) or through store-depleted $Ca^{+2}$-influx (Heemskerk and Sage, 1994), we examined the effect of $Ca^{2+}$ channel blockers (verapamil and diltiazem) on platelet aggregation. The synergistic effect of low concentrations of PAF (40 nM) and 5-HT (2 μM) was markedly inhibited by low concentrations of verapamil and diltiazem with $IC_{50}$ of 5 and 8 μM, respectively (Table 1). In contrast, PKC inhibitor, chelerythrine, had no inhibitory effect, excluding any role of PKC in this cascade (data not shown). These data suggest that platelet aggregation mediated by co-addition of sub-threshold concentrations of these agonists predominantly is $Ca^{2+}$-dependent and also occurs through influx of calcium through receptor-operated calcium channels.

PAF is considered to be a potent activator of TXA2 formation through activation of cyclooxygenase-1 (COX-1). To examine if these two agonists show synergism on COX-1 activity, we measured TXA2 formation in agonist-treated platelets. Similar to its effect on platelet aggregation, 5-HT markedly potentiated the TXA2 formation

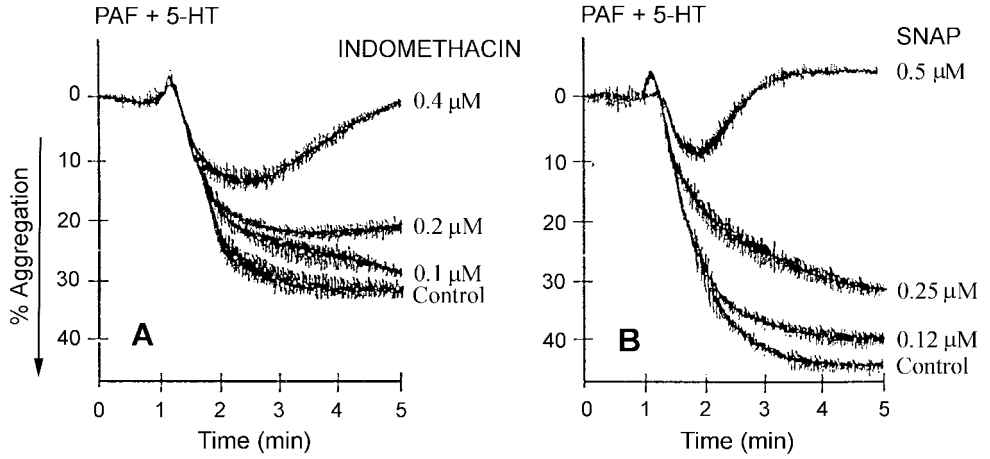


**Figure 3.** (A) Cyclooxygenase (COX) inhibitor, indomethacin, (B) Nitric oxide donor, SNAP, inhibits platelet aggregation induced by co-addition of sub-threshold concentrations of 5-HT and PAF. Inhibitors were added one min before the addition of agonists. Control means platelet aggregation induced by 5-HT (2 μM) plus PAF (40 nM) (n=7).

by low concentrations of PAF (40 nM) as shown in Figure 2. Moreover, the selective COX-1 inhibitor, indomethacin, completely blocked platelet aggregation at very low concentrations ($IC_{50}$=0.25 μM) suggesting the potential involvement of COX-1 in this synergism (Figure 3A). Recent studies indicate an important role of nitric oxide (NO) in modulating platelet aggregation (Shah *et al*., 1999). An analysis of results show that NO donor, SNAP, completely blocked platelet aggregation mediated by synergistic interaction of PAF and 5-HT (Figure 3B). These data provide evidence in support of an important role for NO in negatively modulating the human platelet aggregation.

Agonist-stimulation of $G_q$/PLC/$Ca^{+2}$ cascade leads to activation of mitogen-activated protein (MAP) kinases (Della Roca *et al*., 1999; Heemskerk and Sage, 1994). We have recently shown that synergism of adrenaline and histamine involves phosphorylation of extracellularly regulated MAP kinases (Shah *et al*., 2000). Results in Figure 4A show that PAF stimulated the phosphorylation of ERK1/2 and 5-HT increased this effect. Pretreatment

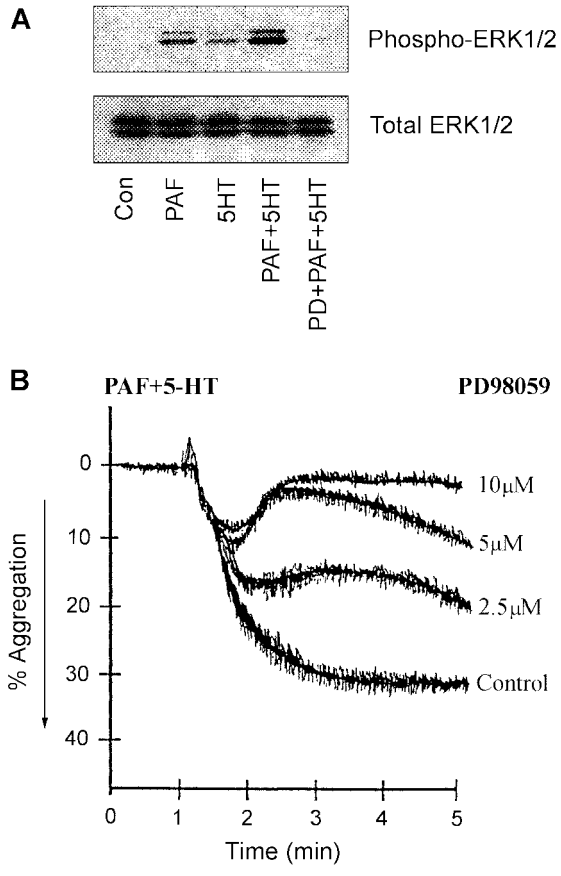


**Figure 4.** (A) Effects of 5-HT and PAF on the phosphorylation of extracellularly regulated MAP kinases (ERK1/2) in human platelets. PD98059 blocks the ERK1/2 activation. (B) Concentration-dependent effects of MEK inhibitor, PD98059, on platelet aggregation induced by co-addition of 5-HT and PAF. Control means platelet aggregation induced by 5-HT (2 μM) plus PAF (40 nM) (n=6).

of PRP with MEK inhibitor, PD98059, inhibited ERK1/2 activation induced by co-addition of sub-threshold concentrations of PAF and 5-HT. Similarly, PD98059 also inhibited platelet aggregation in response to agonist synergism (Figure 4B). Since MEK inhibitor, PD98059, is reported to directly inhibit purified COX-1 and -2 (Borsch-Haubold *et al*., 1998), we examined the effect of PD98059 on arachidonic acid metabolism and $TXA_2$ formation. An analysis of results show that PD98059 also inhibits agonist-induced $TXA_2$ production with an $IC_{50}$ of 5 ± 0.3 μM. Thus, it is possible that the inhibitory effect of PD98059 on platelet aggregation is mediated through inhibition of COX activity. We also examined the effect of inhibitors against PAF alone and the data is given in Table 1. Our results show that inhibitors of various signalling pathways inhibited PAF plus 5-HT induced aggregation at lower $IC_{50}$ values as compared to the $IC_{50}$ values obtained against PAF alone (Table 1).

Phosphatidylinositol 3-kinase (PI 3-kinase), is activated by GPCRs and growth factors, and plays an important role in platelet aggregation. The selective inhibitor of PI 3-kinase, wortmannin, is reported to block the aggregation response induced by synergism of 5-HT and adrenaline (Shah and Saeed, 1995). Our results show that wortmannin inhibited platelet aggregation ($IC_{50}$=620 nM) induced by the synergism of PAF and 5-HT (Table 1).

## Discussion

Most of the platelet agonists, which interact with G-protein coupled receptors, exert their effects through activation of either $G_q$/PLC (*e.g.* PAF, thrombin) or $G_i$/adenylyl cyclase (*e.g*., adrenaline) in platelets (Siess *et al*., 1989; Brass *et al*., 1993). The second messengers, $Ca^{2+}$ and PKC generated in response to $G_q$/PLC activation bring about coordinated changes leading towards platelet aggregation (Crabos *et al*., 1992; Heemskerk and Sage, 1994). The platelet aggregation by sub-threshold concentrations of PAF and 5-HT were inhibited by receptor antagonists, and inhibitors of PLC and MAP kinase and Cox. We (Shah *et al*., 1999; 2000), and others have recently shown that concomitant activation of $G_i$ and $G_q$ protein-linked signalling pathways results in aggregation of human platelets. However, the present data demonstrate that activation of $G_q$ protein by two different agonists at sub-threshold concentrations is equally potent in eliciting the aggregation response by platelets.

In platelets, PAF and 5-HT cause stimulation of $G_q$ protein followed by activation of PLC. This explains why U73122, a selective inhibitor of PLC, shows strong inhibitory effects on platelet aggregation induced by co-activation by these agonists. Further, PLC activation leads to generation of $IP_3$, release of $Ca^{2+}$ from internal

stores and eventually store-depleted $Ca^{2+}$ influx (Heemskerk and Sage, 1994) that was inhibited by $Ca^{2+}$-channel blockers (verapamil and diltiazem). Moreover, the increase in cytosolic $Ca^{2+}$ causes activation of $PLA_2$ and COX-1 activity, thus stimulating $TXA_2$ formation (Heemskerk and Sage, 1994). Since the synergism of these agonists was inhibited by indomethacin, a COX-1 inhibitor, it seems that the agonist-mediated synergism follows activation of COX-1 distal to PLC/$Ca^{2+}$ activation. We tested if increasing the intracellular nitric oxide (NO) levels by NO donor and thus activating cGMP kinase has any inhibitory effect on platelet aggregation. Our results show that NO donor, SNAP, inhibited platelet aggregation at very low concentrations ($IC_{50}$=0.3 μM) suggesting that PAF and 5-HT synergism is highly sensitive to NO generation in human platelets (Figure 3B). However, the role of PKC in the present study was excluded as PKC inhibition had no effect on the synergism of PAF and 5-HT in platelets.

The cyclic nucleotides, cAMP and cGMP, through activation of cAMP- and cGMP-dependent protein kinases, down-regulate the $Ca^{2+}$ responses and thus inhibit platelet aggregation (Heemskerk and Sage, 1994). In fact platelets are abundant in cAMP and cGMP-dependent protein kinases and these kinases can inhibit PLC-induced $IP_3$ production through inactivation of $IP_3$ and $TXA_2$ receptors (Heemskerk and Sage, 1994; Wang *et al.*, 1998).

The inhibition of PAF/5-HT synergism by MEK inhibitor, PD98059, suggests the involvement of MAP kinase that is known to be distal to Gq/PLC (Della Roca *et al.*, 1997; 1999). PAF through activation of $G_q$/PLC is reported to activate ERK1/2 MAP kinases through different signalling pathways that include PI 3-kinase, tyrosine kinases such as proline-rich tyrosine kinase (Pyk2), Ras/Raf and MEK1/2 (Ishii and Shimizu, 2000; Miike *et al.*, 2000). ERK1/2 phosphorylation can activate $cPLA_2$ leading to production of prostaglandins and $TXA_2$ through activation of COX (Ishii and Shimizu, 2000). In fact, cPLA2 is also a potential target of activation by increase in cytosolic $Ca^{2+}$. Taken together, it appears as both $Ca^{2+}$ and MAP kinases play an important role during synergistic interaction of 5-HT and PAF in human platelets.

The selective MEK inhibitor, PD98059, is also known to inhibit the COX-1 and -2 activities (Borsch-Haubold *et al.,* 1998; McNicole *et al.*, 1998). Using purified COX-1 and -2, Borsch-Haubold *et al.*, (1998) showed that PD98059 inhibited arachidonic acid metabolism and $TXA_2$ formation at quite low concentrations ($IC_{50}$=0.8 μM). These authors reported that higher concentrations of PD98059 were required to inhibit platelet aggregation induced by arachidonic acid, thrombin and collagen. Under our experimental conditions, PD98059 inhibited both $TXA_2$ production and platelet aggregation with $IC_{50}$ of 5 and 3 μM, respectively (Figures 4A & B). Therefore, the possibility that inhibition of agonist-induced platelet aggregation by PD98059 is due to blockade of COX activity cannot be ignored.

Our previous studies have shown an important role of PI 3-kinase in 5-HT mediated potentiation of platelet aggregation by adrenaline (Shah and Saeed, 1995). In addition, the inhibitors of PI 3-kinase block platelet aggregation induced by low, but not high, concentrations of PAF (Lauener *et al.*, 1999). More recently, PI 3-kinase was shown to be involved in the thrombopoietin (TPO) mediated potentiation of platelet function. TPO stimulated ERK1/2 MAP kinase activation by increasing the association of tyrosine phosphorylated Gab1 with p85 subunit of PI 3-kinase. Our data show that PI 3-kinase inhibitor, wortmannin, abolished platelet aggregation only at higher concentrations (1 μM). Since higher concentrations of wortmannin (>100 nM) are known to inhibit other signaling proteins such as myosin light chain kinase, in platelets, the involvement of PI 3-kinase in this cascade cannot be over-emphasized.

The mechanism of synergism among various platelet agonists is reported to occur due to activation of $Ca^{2+}$ signalling cascade. A rise in $Ca^{2+}$ induced by first agonist primes platelets for an enhanced functional response to the second agonist (Ware *et al.*, 1987; Shah *et al.*, 1999). $Ca^{2+}$ plays pivotal role in platelet aggregation (Heemskerk and Sage, 1994; Shah *et al.*, 1998). Interruption in the process of $Ca^{2+}$ activation either through $Ca^{2+}$ channels (Shah *et al.*, 1998) or $G_q$ proteins can

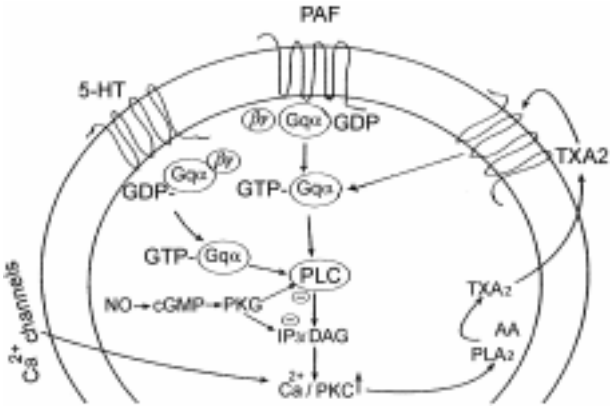


**Figure 5.** Signalling pathways involved in the synergistic interaction of GPCR agonists, 5-HT and PAF. Both agonists activate phosphoinositide-linked G protein (Gq) and phospholipase C (PLC), which leads to generation of inositol triphosphate ($IP_3$) and diacylglycerol (DAG), and thus release of $Ca^{2+}$ from internal stores and activation of protein kinase C (PKC). The agonist-induced store-depletion of $Ca^{2+}$ also increases $Ca^{2+}$ influx through membranes. Both $Ca^{2+}$ and PKC are involved in the release of granule contents and activation of phospholipase A2 ($PLA_2$), thus generation of potent agonist, thromboxane $A_2$ ($TXA_2$), which in an autocrine fashion acts on platelets through Gq protein. Nitric oxide (NO) inhibits platelet aggregation through production of cyclic GMP (cGMP) from guanylate cyclase. The cGMP activates protein kinase G (PKG) that exerts inhibitory effect on platelet aggregation through phosphorylation of PLC, $Ca^{2+}$-channels or $IP_3$-receptor.

interfere with the activation of platelets. Offermanns *et al.* (1999) showed that $G_q$ protein-deficient mice lack the ability of platelet aggregation. Co-activation of PAF and 5-HT receptors on platelets seems to follow the $G_q$/PLC/$Ca^{2+}$, and inhibitors of PLC, MAP Kinase and COX, (Figure 5). The synergism among various platelet agonists in the body is of great clinical significance as it can lead to marked potentiation of the platelet activation and thus alter the cardiovascular physiology. In conclusion, our studies show that sub-threshold concentrations of 5-HT potentiate the platelet aggregation mediated by PAF *in vitro*, and this synergism is negatively modulated by nitric oxide donor, SNAP, suggesting a potential regulatory role of nitric oxide in platelet function.

## Acknowledgements

This study was supported by the research funds from the Aga Khan University, Karachi.

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